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SHORT COMMUNICATION

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Barley isochorismate synthase mutant is phylloquinone-deficient, but has normal basal salicylic acid level

Yuan Qin[a], Anna Maria Torp[a], Gaëtan Glauser[b], Carsten Pedersen[a], Søren K. Rasmussen [a], and Hans Thordal-Christensen[a]

[a]Department of Plant and Environmental Sciences, Copenhagen Plant Science Centre, University of Copenhagen, Copenhagen, Denmark; [b]Institute of Biology, Neuchâtel Platform Analytical Chemistry, University of Neuchâtel, Neuchâtel, Switzerland

ABSTRACT

Salicylic acid (SA) is an important signaling hormone in plant immunity. It can be synthesized by either the phenylpropanoid pathway or the isochorismate pathway, but mutant studies of this have been scarce in other species than Arabidopsis. Here we identified a mutation that introduced a stop-codon early in the barley gene for isochorismate synthase (ICS). We found that homozygous *ics* plants wilted if not sprayed with 1,4-dihydroxy-2-naphthoic acid, a precursor of phylloquinone, also synthesized via the isochorismate pathway. Interestingly, *ics* had unchanged SA, suggesting that the basal level of SA is synthesized via the phenylpropanoid pathway. Previous studies have failed seeing increased SA levels in barley after attack by the powdery mildew fungus, *Blumeria graminis* f.sp. *hordei* (*Bgh*), and indeed, we saw no changes in the interaction of *ics* with this fungus. Overall, we hope this mutant will be useful for other studies of SA in barley.

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Plants have a highly advanced immune system, which efficiently resist most pathogens. After plant receptors have recognized pathogen cues, a sophisticated signaling network activates transcriptional and cellular reprogramming that prevents pathogens from causing disease. This includes expression of many antimicrobial proteins and secondary metabolites, buildup of cellular structures to prevent pathogen ingress and activation of a localized programmed cell death reaction, referred to as the hypersensitive response (HR).[1] A central compound in signaling for immunity is salicylic acid (SA). It activates many downstream response genes, e.g. encoding pathogenesis-related proteins, and besides it has a function as a general amplifier of immunity.[2] SA accumulates in many plants after pathogen attack, and RNA-seq data suggest that it stimulates immunity through its activation of a large number of key regulator genes.[3] The plant ubiquitous protein, NPR1, plays a central role as an SA sensor that regulates transcriptional reprogramming in immunity after it becomes re-localized to the nucleus where it interacts with a number of transcription factors.[4]

SA is synthesized by two biosynthetic pathways, the isochorismate pathway and the phenylpropanoid pathway.[2] In Arabidopsis, basal and pathogen-induced levels of SA are primarily derived from the isochorismate pathway, albeit the phenylpropanoid pathway also contributes. This was shown in plants mutated in the genes encoding isochorismate synthase 1 (ICS1, also named SA induction-deficient 2, SID2), ICS2 and four genes encoding phenylalanine ammonia-lyase.[4–8] However, the use of mutants to study the two pathway's contribution to SA biosynthesis has been very sparse in other plants. Here this question instead has been addressed using RNA interference.[9] For instance, virus-induced gene silencing of *ICS* in *Nicotiana benthamiana* suggested that pathogen-induced SA is derived from isochorismate.[10]

The importance of SA in Poaceae species is less clear than in dicot species, and the contribution of the two biosynthesis pathway is not well studied. Even though SA appears involved in Arabidopsis resistance to bacterial and powdery mildew fungal pathogens,[5,11,12] it does not accumulate in barley in response to such pathogens.[13,14] Yet, a recent study showed that barley (*Hordeum vulgare*) leaves inoculated with *Fusarium graminearum* had a 30-fold increase in the SA level, which, in an *ICS* RNAi transgenic line, was reduced to basal level.[15] Interestingly, the same study showed that the *ICS* RNAi line has decreased post-invasive immunity to *Bgh*, which is unexpected according to Hückelhoven et al.[13] A study of maize attacked by *Ustilago maydis* has revealed that a fungal chorismate mutase effector contributes to fungal virulence after transfer to cells of the plant, where it reduces the SA level.[16] This strongly advocates for the importance of SA, synthesized by the isochorismate pathway, in immunity in maize.

In the present work, we aimed at mutant studies of SA in barley. This species has a single gene encoding isochorismate synthase (ICS).[15] To search for mutations in this gene, a cultivar Golden Promise mutant population was obtained after treatment with fast neutrons in a 12–18 Gy dose. The resulting M_2 seeds were mutagenized again by steeping in

CONTACT Hans Thordal-Christensen htc@plen.ku.dk Department of Plant and Environmental Sciences, Copenhagen Plant Science Centre, University of Copenhagen, Copenhagen, Denmark

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1.5 mM sodium azide for 2½ h before soaking in tap water for 2 h. The seeds were subsequently dried and sown in the field. M_2 seed samples were harvested separately from 2,132 single plants, and one seed from each sample was germinated for DNA extraction. The resulting DNA samples were screened by TILLING according to de Bang et al.[17] This led to the identification of a heterozygous line, *ICS ics*, where codon 153 (TGG, encoding tryptophan) was changed into the stop-codon, TGA. *ICS* has several exons. However, transcript sequence data indicate that only one splice variant exits, and it contains codon 153 (https://ics.hutton.ac.uk/barleyrtd/blast_page.html). One-fourth of the offspring resulting from the selfing of *ICS ics* started wilting ~10 days after emergence (Figure 1), and genotyping of 84 wilting and 238 non-wilting plants demonstrated complete co-segregation of *ics* homozygosity and wilting.

Garcion et al.[7] previously found the Arabidopsis double mutant *ics1 ics2* to be completely deficient of phylloquinone, which is essential for electron transfer in photosystem I. Isochorismate is an early precursor for phylloquinone, synthesized via 1,4-dihydroxy-2-naphthoic acid (NA) in a multistep pathway.[18,19] Thus, we were able to rescue the barley *ics* plants to almost wild-type size by spraying with NA (Figure 1). *ICS* is located on barley chromosome 5, whereas the genes, encoding the enzymes mediating the biosynthetic steps between isochorismate and NA,[19] are located on barley chromosomes 3, 4, 6 and 7. Thus, the rescuing by NA, together with the co-segregation of *ics* and wilting, confirms that the *ics* mutation prevents isochorismate synthesis.

Next, we analyzed the content of phylloquinone and SA in homozygous *ics* and *ICS*, segregating from *ICS ics*, as well as in wild-type Golden Promise. Phylloquinone was undetectable in *ics* and indifferent in *ICS* and wild-type, while all three samples had approximately 7 ng SA/g fresh weight (Figure 2).

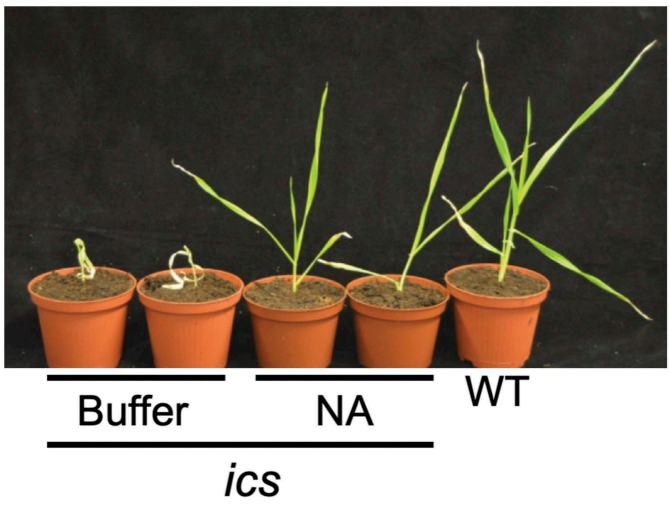

Figure 1. Wilting of *ics* is overcome by spraying with 1,4-dihydroxy-2-naphthoic acid (NA). Four 18-day-old *ics* plants sprayed with 0.1 mM NA or buffer (0.1% dimethyl sulfoxide, 0.1% Tween-20) every other day from day 7 after sowing. WT, Golden Promise. Homozygous *ics* plants were selected by genotyping using the allele-specific primers, ICS-F 5′-TGGCCACAAGGAGCAGTAG-3′, ics-F 5′-GTGGCCACAAGGAGCAGTAA-3′, and reverse primer, ICS-R 5′-TCACTGAACTC AACCTGCAAA-3′, in a touch-down PCR reaction in which the annealing temperature was reduced from 74ºC to 66ºC over the first eight cycles.

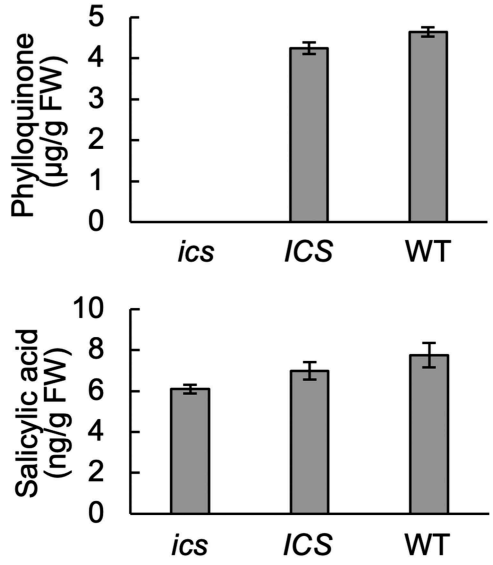

Figure 2. Phylloquinone, but not SA, is reduced in *ics*. Analyses were made by UHPLC-MS/MS according to Glauser et al.[20] Leaves from homozygous *ICS* and *ics* plants were selected by analyzing DNA from leaf tips as in Figure 1. Each sample consisted of two first leaves from 10-day-old barley plants. Error bars, SE; n, 6 (phylloquinone) and 8 (SA).

This indicated that basal SA is not derived from the isochorismate pathway in barley.

Despite no induction of the SA-level has been observed after inoculation with *Bgh*,[13] Hao et al.[15] observed that *ICS* RNAi lines had decreased immunity to this pathogen. This spurred us to re-test this observation, as it cannot be ruled out that a minor localized SA increase, not detectable in whole leaf extracts, can have significance. Therefore, genotyped *ics* and *ICS* plants, segregating from *ICS ics*, were inoculated with *Bgh* and the rate of penetration, secondary hyphal growth and host cell death were estimated (Figure 3). However, no significant differences between *ics* and *ICS* plants were revealed.

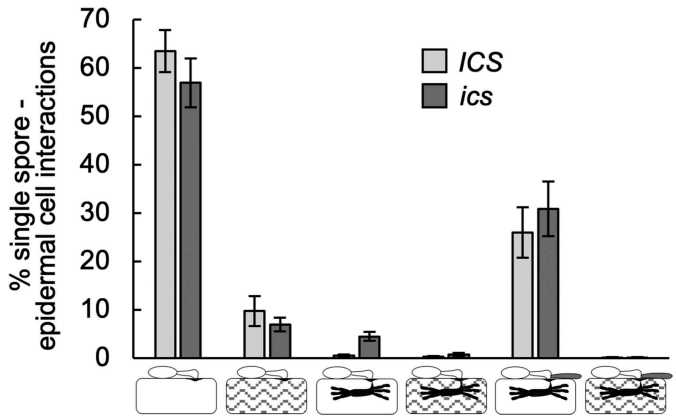

Figure 3. *ics* has a normal interaction with the powdery mildew fungus. Two days after inoculation with the virulent *Bgh* isolate, DH14, leaf tips were cut for genotyping (see Figure 1), and the remaining leaf parts were trypan blue stained for microscopy. Five leaves (repeats) were assessed from each genotype, and the outcome of 110 to 210 single spore interactions was scored from each leaf according to Zhang et al.[21] Error bars, SE.

In summary, we have identified a barley *ics* knock-out allele, which when homozygous confers plant wilting. This phenotype could be rescued by spraying with NA, confirming that the mutant is affected in the isochorismate pathway. Yet, we were unable to observe a change in the SA content of *ics*, suggesting that basal SA in barley is produced by the phenylpropanoid pathway. As anticipated from previous failures to see the induction of SA levels in barley in response to *Bgh*,[13] we observed no altered interaction of *ics* with this pathogen. Yet, we hope this mutant will become useful for studies of signaling in immunity in barley.

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ORCID

Søren K. Rasmussen http://orcid.org/0000-0001-6169-2504

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